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AWAPATENT

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MONOCLONAL ANTIBODY

Field of the Invention

The present invention relates to a monoclonal anti-
body suitable for monitoring the activity of systems in-
volving protein C inhibitor, a method for preparation of
5   said monoclonal antibody, a method for monitoring the
activity of systems involving protein C inhibitor and a
method for diagnosis of *e.g.* venous thrombosis, wherein
said monoclonal antibody is utilised.

Background of the Invention

10   Protein C is a proenzyme to "activated protein C".
Activated protein C, hereinafter denoted APC, is a serine
proteinase, and its proenzyme protein C is a liversynt-
hesised glycoprotein having 461 amino acid residues and a
molecular weight of approximately 62 kDa. Protein C
15   carries two parts denoted the light and the heavy chain,
respectively (formed by limited proteolysis of a single
chain precursor). The light chain is glycosylated on its
Asn 97 residue, whereas the heavy chain is glycosylated
on its Asn 248, Asn 313 and Asn 329 residues (Asn 329
20   being glycosylated only in so-called "single chain pro-
tein C"). Of all the protein C in plasma, about 85% con-
tains both the light and heavy chain, whereas about 15%
consists of single chain protein C. There is no known
difference in biological activity between these two forms
25   of protein C.

The light chain of protein C contains an amino-ter-
minal domain with nine γ-carboxyglutamic acid (Gla) resi-
dues, which have been formed by vitamin K-dependent car-
boxylations. This domain is followed by two domains, both
30   of which are homologous to the epidermal growth factor
(EGF) precursor. Of said two domains, one has a single
hydroxylated aspartic acid residue, *i.e.* consists of

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erythro-β-hydroxyaspartic acid. The light and the heavy chains are linked together via one disulfide bond only.

Moreover, protein C requires vitamin K for its normal biosynthesis, and it circulates in blood plasma at a
5   concentration of approximately 4 mg/l.

Protein C is an important regulator of blood coagulation, and it is activated by thrombin complexed with thrombomodulin. This activation involves removal of an activation peptide with 12 amino acids from the amino-
10   terminal end of the heavy chain of protein C, whereby APC is formed. APC is a typical serine proteinase with high arginyl bond specificity.

The substrates degraded by APC are the cofactors known as factor V/V_a and factor $VIII/VIII_a$, respectively,
15   where "$_a$" denotes the active form of the cofactor. In coagulation pathways, the factor $VIII_a$ forms a membrane-bound complex with the factor IX_a, whereby the coagulation factor X is activated to a serine proteinase denoted coagulation factor X_a. Furthermore, the factor V_a
20   forms a membrane-bound complex with the coagulation factor X_a, whereby prothrombin is activated to thrombin.

The degradation of the factors V_a and $VIII_a$ by APC requires two cofactors. These are protein S, which is a vitamin K-dependent protein, and factor V, which is the
25   unactivated form of factor V_a. The degradation results in cleavage of the factors V_a and $VIII_a$, whereby their affinity for factors X_a and IX_a, respectively, is reduced. Thus, said cleavage results in a reduction of the rate of formation of both thrombin and coagulation factor X_a. In
30   other words, the blood coagulation cascade is turned off.

As other serine proteinases, APC has its inhibitors. These are protein C inhibitor (hereinafter denoted PCI; plasma concentration approximately 4 mg/l), $α_1$-proteinase inhibitor (hereinafter denoted $α_1$-antitrypsin; plasma
35   concentration approximately 1 g/l) and $α_2$-macroglobulin (plasma concentration approximately 2 g/l). PCI and $α_1$-antitrypsin belong to a group of inhibitors sometimes

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referred to as serpines (<u>ser</u>ine <u>p</u>roteinase <u>in</u>hibitors),
and form 1:1 complexes with APC.

The complex formation between APC and PCI proceeds
at a slow rate, and it proceeds even slower in respect of
5   α_1-antitrypsin. This slow rate is manifested by a long
half-life for APC in plasma, wherein $t_{1/2}$ is about 20 min.
However, the rate of complex formation between APC and
PCI is increased by heparin.

Upon complex formation with APC, the serpin is
10 cleaved in its so-called bait region, whereby a stable
intermediate acyl complex is formed. With time, the in-
termediate acyl complex dissociates, whereby APC is re-
generated and a proteolytically modified, i.e. cleaved,
inactive serine proteinase inhibitor is formed.

15   The intermediate acyl complexes are removed rather
rapidly from the circulatory system. $T_{1/2}$ in baboons is
approximately 40 and 140 min for the APC:PCI and
APC:α_1-antitrypsin complexes, respectively. PCI appears
to be the prime inhibitor for APC with α_1-antitrypsin and
20 α_2-macroglobulin taking over as the PCI concentration is
substantially lowered.

PCI also inhibits thrombin and factor X_a, both of
which are blood coagulation proteinases. In addition, PCI
inhibits trypsin, chymotrypsin, urokinase plasminogen
25 activator (uPA), tissue type plasminogen activator (tPA),
plasma kallikrein, factor XI_a, prostatic specific antigen
(PSA) and the prostata specific kallikrein-like serine
proteinase denoted HGK1.

The biological importance of APC can be illustrated
30 in several ways, e.g.

   i)   newborn children with homozygous protein C
        deficiency die during the first days of life in
        a clinical condition denoted purpura fulminans,
        which is the result of widespread thromboti-
35   sation of capillaries;

   ii) heterozygosity for protein C deficiency has in
        many cases been associated with a thrombofilia

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that accounts for approximately 2% of all
hereditary thrombofilia;

iii) a frequently occurring (present in 2-10% of
caucasian populations) point mutation in
factor V, rendering factor V/V$_a$ resistant to
degradation by APC, is associated with a mild
form of hereditary thrombofilia in the
heterozygous form and a more severe form of
thrombofilia in the homozygous form;

iv) disseminated intravascular coagulation is
associated with a depletion of activated
protein C, a process which often leads to a
fatal outcome;

v) gram negative septicemial (caused by e.g.
meningococci) leads to a depletion of activated
protein C, a condition shown to be fatal in
baboons as well as in humans.

In summary, the considerable biological importance
of serine proteinases, particularly protein C, as regu-
lators of blood coagulation as well as their role as key
proteins in the so-called protein C anticoagulant pathway
has resulted in a great need in the art to develop power-
ful research tools and extract clinically relevant infor-
mation from e.g. the concentration of APC in plasma.

In particular, such clinically relevant information
could be very useful in the diagnosis of e.g. venous or
arterial thrombosis, including coronary infarction or
pulmonary embolism.

Prior Art

To serve the above-mentioned purposes and measure
the activation of anticoagulant systems involving protein
C, numerous methods have been developed, as set forth be-
low.

In Bauer, K.A., Kass, B.L., Beeler, D.L., Rosenberg,
R.D., *J. Clin. Invest.*, **74**:2033-2041 (1984), a method is
disclosed, wherein the plasma concentration of the acti-
vation peptide removed from protein C (*vide supra*) is

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measured. Since said activation peptide has a very high
renal clearance, its plasma concentration is low. Thus,
this method is cumbersome as it requires large amounts of
plasma.

5   In Gruber, A., Griffin, J.H., *Blood*, **79**:2340-2348
(1992), a method is disclosed, wherein the plasma con-
centration of APC is measured. Here, an ELISA technique
with a catching monoclonal antibody against protein C was
used. Since said catching monoclonal antibody does not
10   inhibit the activity of APC, an amidolytic assay was
used. However, due to the low plasma concentration of
APC, the incubation time was generally very long (up to
days) and impractical.

         In an approach disclosed in Espana, F., Zuazu, I.,
15   Vicente, V., Estelles, A., Marco, P., Aznar, J., *Thromb.*
Haemost., **75**:56-61 (1996), heparin-mediated enhancement
of the rate of complex formation between APC and PCI is
utilised. This method requires the presence of *e.g.* he-
parin, benzamidine and D-phenylalanyl-L-prolyl-L-arginine
20   chloromethyl ketone (PPAC).

         However, the most relevant approaches disclosed re-
late to methods, wherein the concentration of a complex
between APC and PCI, or between APC and α_1-antitrypsin,
is measured by use of monoclonal antibodies. These ap-
25   proaches are all based on the assumption that said con-
centration reflects the concentration of APC, and hence
also the degree of activation of the protein C anticoa-
gulant system. Here, a catching monoclonal antibody
raised against either APC or PCI is used together with a
30   suitable tracer agent in an ELISA technique.

         Thus, if the catching monoclonal antibody is speci-
fic for PCI, the tracer agent could be an antibody speci-
fic for an epitope in protein C, or vice versa. However,
an inherent problem in this approach is that the concen-
35   tration of free, uncomplexed PCI and APC, respectively,
is very high in comparison with the APC:PCI complex to be
measured. As an example, due to competitive binding be-

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tween free, uncomplexed PCI and complex-bound PCI to a
catching monoclonal antibody raised against PCI, the
sensitivity of such an approach is greatly reduced.
Typical such approaches are disclosed in JP 02-236 452,
5   Espana, F., Griffin, J.H., *Thromb. Res.*, **55**:671-682
(1989) and Espana, F., Vicente, V., Scharrer, I.,
Tabernero, D., Griffin, J.H., *Thromb. Res.*, **59**:593-608
(1990).

    In an approach disclosed in Minamikawa, K., Wada,
10   H., Wakita, Y., Ohiwa, M., Tanigawa, M., Deguchi, K.,
Hiraoka, N., Huzioka, H., Nishioka, J., Hayashi, T.,
Thromb. Haemost., **71**:192-194 (1994), a precipitated
barium salt is used to bind PCI in complex with APC,
whereas uncomplexed PCI does not bind to the precipitated
15   barium salt. After centrifugation and dissolution in EDTA
(aq), the sample is subjected to ELISA, wherein a
catching monoclonal antibody raised against PCI is used.
Although useful from a sensitivity point of view, this
approach is time-consuming as well as laborious.

20   In Laurell, L., Carlson, T.H., Stenflo, J., *Thromb.
Haemost.*, **60**:334-339 (1988), it is disclosed monoclonal
antibodies raised against cleaved PCI, wherein said
monoclonal antibodies are applied in an assay for mea-
suring the plasma concentration of APC:PCI complexes. All
25   of said monoclonal antibodies also had specific affinity
for uncleaved, uncomplexed PCI.

    In summary, there is still a great need in the art
for a monoclonal antibody with high specific affinity for
a serine proteinase inhibitor, *e.g.* PCI, in complex with
30   a serine proteinase, *e.g.* APC, where said monoclonal
antibody has substantially no affinity for PCI in its un-
complexed form.

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Disclosure of the Invention

According to the invention, there is now provided a
new monoclonal antibody which overcomes the problems in
the art referred to above. The monoclonal antibody ac-
5   cording to the invention is suitable for monitoring the
activity of systems involving protein C inhibitor, such
as anticoagulant systems, and said monoclonal antibody is
characterised by having specific affinity for both

        i)   a complex between a serine proteinase and
10   an inhibitor thereof, and

        ii) a cleaved form of said inhibitor,

while having substantially no specific affinity for said
inhibitor in its uncleaved and uncomplexed form;
or being a derivative thereof having the same biological
15   activity.

The monoclonal antibody according to the present in-
vention is obtainable by immunisation of an animal with a
mixture of

        i)   a complex between a serine proteinase and
20   an inhibitor thereof, and

        ii) a cleaved form of said inhibitor,

followed by screening for and isolation of said mono-
clonal antibody. Said animal is preferably a mouse, most
preferably a Balb/c mouse.

25   Preferably, said serine proteinase is selected from
the group consisting of activated protein C (APC),
thrombin, coagulation factor X_a, trypsin, chymotrypsin,
urokinase plasminogen activator (uPA), tissue type plas-
minogen activator (tPA), plasma kallikrein, factor XI_a,
30 · HGK1 and prostatic specific antigen (PSA).

As said inhibitor, either protein C inhibitor (PCI)
or α_1-antitrypsin is preferred.

In the most preferred embodiment, the monoclonal
antibody according to the invention is obtainable, or
35   obtained, by immunisation with a *mixture* of cleaved PCI
and PCI in complex with PSA.

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In another aspect, the present invention also re-
lates to a method for preparation of a monoclonal anti-
body as defined above, wherein an animal is immunised
with a mixture of

5   i) a complex between a serine proteinase and
                   an inhibitor thereof, and
               ii) a cleaved form of said inhibitor,
followed by screening for and isolation of said mono-
clonal antibody.

10   In yet another aspect, the present invention relates
to a method for monitoring the activity of anticoagulant
systems involving protein C, wherein a monoclonal anti-
body as defined above is used in an immunoassay.

        Preferably, said immunoassay comprises a sandwich-
15 type immunoassay. More preferably, said sandwich-type
immunoassay is a DELPHIA®, ELISA or magnetic bead tech-
nique comprising a tracer agent and said monoclonal anti-
body bound to a surface.

        In an embodiment of said method, said tracer agent
20 comprises an antibody having specific affinity for said
serine proteinase or an epitope shared by said serine
proteinase and said inhibitor.

        In another embodiment, said tracer agent is con-
jugated to a suitable enzyme and/or labelled with a
25 tracing substance. Said enzyme is e.g. an alkaline
phosphatase, horse radish peroxidase or a β-galactosi-
dase. Said tracing substance is e.g. ^{125}I, ^{131}I, Eu^{3+} or
Sm^{3+} or a similar lanthanide.

        In a further aspect, the present invention also
30 relates to a method for diagnosis of venous thrombosis,
arterial thrombosis, embolism, coronary infarction, dis-
seminated intravascular coagulation or disorders in-
volving lupus anticoagulants, wherein a monoclonal anti-
body as defined above is utilised.

35   Furthermore, this invention relates to a method for
diagnosis of venous thrombosis, arterial thrombosis, em-
bolism, coronary infarction, disseminated intravascular

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coagulation or disorders involving lupus anticoagulants,
wherein a monitoring method as defined above is utilised.

Moreover, the present invention relates to the use
of a monoclonal antibody as defined above for in vitro
5   diagnosis of venous thrombosis, arterial thrombosis, em-
bolism, coronary infarction, disseminated intravascular
coagulation or disorders involving lupus anticoagulants.

Also, this invention concerns the use of a moni-
toring method as defined above for in vitro diagnosis of
10   venous thrombosis, arterial thrombosis, embolism, coro-
nary infarction, disseminated intravascular coagulation
or disorders involving lupus anticoagulants In yet a-
nother aspect, the present invention relates to a kit for
qualitative or quantitative determination of the activity
15   of systems involving protein C inhibitor comprising a
monoclonal antibody as defined above.

Thus, the present invention allows for precise quan-
titative measurements of complexes between e.g. human APC
and PCI in blood plasma. Indeed, the present invention
20   encompasses measurements of complexes between PCI and
thrombin or any other serine proteinase known to form
complexes with PCI, e.g. coagulation factor X_a (this em-
bodiment requires the exchange of the tracer agent only).

Some of the incentives for the use of a mixture of
25   cleaved and complexed serine proteinase inhibitor for
immunisation were gathered from mainly three sources.
Firstly, it has been reported that certain monoclonal
antibodies against the serpins antithrombin and C-1
esterase inhibitor (see e.g. de Agostini, A., Patston,
30   P.A., Marottoli, V., Carrel, S., Harpel, P.C., Schapira,
M., J. Clin. Invest., 82:700-705 (1988) and Asakura, S.,
Matsuda, M., Yoshida, N., Terukina, S., Kihara, H., J.
Biol. Chem., 264:13736-13739 (1989)) are more or less
selective for the complexed form of the inhibitor, albeit
35   no such antibodies have been identified against PCI.
Secondly, it has been disclosed in e.g. Björk, I.,
Nordling, K., Olson, S.T., Biochemistry, 32:6501-6505

(1993) that thrombin:antithrombin complexes and anti-
thrombin cleaved from such complexes share epitopes.
These epitopes are not present on native antithrombin.
Thirdly, it has been reported by e.g. Espana, F. and
5   Griffin, J.H. (*vide supra*) that human seminal plasma has
a PCI concentration that is about 50-fold higher in com-
parison with its blood plasma concentration. However, the
PCI present in human seminal plasma is unable to form
complexes with APC. Indeed, the PCI present in human
10   seminal plasma is the cleaved form of PCI.

The monoclonal antibody according to the present
invention, when used as catching antibody in e.g. a
sandwich assay, has very high specific affinity for PCI
in complex with APC as well as for the proteolytically
15   cleaved PCI, but has substantially no or very low speci-
fic affinity for native PCI, *viz* PCI in its uncleaved and
uncomplexed form. In other words, the native PCI has no
influence on the binding of APC:PCI complexes to a sur-
face having the monoclonal antibody according to the pre-
20   sent invention bound thereto. Thus, there is substan-
tially no competitive binding of the native PCI to said
surface, whereby the sensitivity of the method according
to the invention is high.

The method according to the present invention has
25   been devised such that it can easily be automated and
adopted for use in modern automated laboratory instrumen-
tation using e.g. magnetic beads coated with the mono-
clonal antibody of the invention.

The present invention is applicable both as a power-
30   ful research tool and in conjunction with diagnosis of
e.g. venous thrombosis, embolism and coronary infarction.
In particular, the latter application could be very use-
ful as it may detect coronary infarction much earlier
than existing methods, most of which measure the con-
35   centration of enzymes or other proteins released from the
damaged myocardium (CK-MB, troponin T or myoglobin). The
currently used methods are not diagnostically helpful

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until coronary ischemia has already damaged the myocar-
dium. Detectable levels of CK-MB and troponin T are
usually not present until 3-5 h (2-3 h for myoglobin)
after the infarction, depending on its size.

5   Furthermore, the present invention may also help in
the differentiation between coronary infarction and un-
stable angina, thereby improving current diagnostic
procedures most significantly.

       In a sandwich-type immunoassay, such as a DELPHIA®
10 technique, two monoclonal antibodies are used. According
to a preferred embodiment of the present invention, one
has specific affinity for PCI, and this monoclonal anti-
body is bound to a surface and denoted "catcher" in a
DELPHIA® context. The other monoclonal antibody has
15 specific affinity against protein C and is denoted
"tracer agent" in a DELPHIA® context. According to the
present invention, the catcher has been carefully
selected to circumvent the problems associated with the
prior art (vide supra), i.e. that the large molar excess
20 (normally more than 1000-fold) of uncomplexed protein, be
it protein C or PCI, competes with the complexes to be
measured by binding to a surface-bound monoclonal anti-
body.

       Thus, in a preferred embodiment, the inventor has
25 generated mouse monoclonal antibodies which have very
high specific affinity for complexed PCI and cleaved PCI,
but substantially no affinity for native PCI, viz PCI in
its uncleaved and uncomplexed form. Such monoclonal anti-
bodies are used as catcher in an immunoassay, as set
30 forth below, and they were obtained by immunising Balb/c
mice with a mixture of cleaved PCI and PCI in complex
with a serine proteinase known as prostatic specific
antigen (PSA). Furthermore, the inventor has devised
approaches for carefully selecting, i.e. screening and
35 isolating, such monoclonal antibodies, as is disclosed
hereinbelow.

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The present invention is further illustrated by the
following non-limiting examples together with the accom-
panying figures.

Description of the figures

5   Fig. 1 shows affinity chromatograms for APC-com-
plexed (A), cleaved (B) and native (C) PCI, respectively,
obtained on an Affi Gel® 10 column (0,5 x 10 cm), onto
which the monoclonal antibody M36 was immobilised. In
each one of the samples A, B and C, 30 µg of the resp-
10   ective PCI analyte was chromatographed. The column was
equilibrated with 50 mM Tris-HCl/0,5 M NaCl (pH 7,5). The
flow rate was 0,1 ml/min. Bound protein was eluted with
0,1 M glycine-HCl/0,5 M NaCl with pH 2,7 (indicated by
the arrow). The continuous line represents absorbance,
15   whereas the "o-o"-line represents fluorescence. The early
eluting peak in A consists of UV absorbing low molecular
weight compounds and APC from cleaved complexes.

        Fig. 2 shows measurements in real time of the inter-
action of APC:PCI complexes and cleaved PCI with the
20   monoclonal antibody M36 using the surface plasmon reso-
nance (SPR) technique. A) illustrates the interaction of
APC:PCI complexes with M36, and the concentrations of the
APC:PCI complexes were 17,6 nM (●), 8,8 nM (◆), 4,4 nM
(▲) and 2,2 nM (▼). B) illustrates the interaction of
25   cleaved PCI with M36, and the concentrations of cleaved
PCI were 38,5 nM (●), 7,7 nM (▲), 3,9 nM (▼), 1,9 nM (■)
and 1,0 nM (◆). The lines were fitted to the experimental
data, some of which are denoted by the symbols.

        Fig. 3 depicts dose-response curves for measurements
30   of APC:PCI complexes. Each point represents the mean
value of duplicate measurements. (◆) illustrates APC:PCI
complexes in buffer, whereas (Δ) illustrates APC:PCI com-
plexes in citrated plasma to which benzamidine has been
added to a final concentration of 50 mM. The signal given
35   by APC:PCI complexes prior to the addition of standard
has been subtracted.

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Examples

Preparation of proteins:

Native PCI was purified from human plasma according to a method described in Laurell, M. et al. (vide supra).
5   Cleaved PCI and PCI in complex with PSA were purified from human seminal plasma using the same method.

Protein C was purified from human plasma by adsorption to barium citrate, elution with EDTA, column chromatography on DEAE Sepharose® and use of an im-
10   mobilised monoclonal antibody against protein C (HPC-4) that recognises a calcium-dependent epitope in the N-terminal EGF-like domain, as disclosed in Ohlin, A.K., Stenflo, J., J. Biol. Chem., 262:13798-13804 (1987). Protein C was eluted from the column with 0,1 M sodium
15   acetate/acetic acid buffer containing 0,5 M NaCl (pH 4,0). By addition of concentrated Tris buffer, an immediate adjustment to pH 7,5 was performed.

APC was prepared by thrombin-mediated activation of protein C, followed by isolation of APC by chromatography
20   on an HPC-4 column, as described by Ohlin, A.K. and Stenflo, J. (vide supra).

Complexes used as standard in the method of measuring APC:PCI complexes were made by incubation of APC with a two-fold molar excess of PCI in 50 mM Tris-HCl,
25   0,1 M NaCl, 1 mM EDTA with pH 7,5. The complexes were isolated by affinity chromatography on an HPC-4 column, as described above. The purity of the isolated complex was established by SDS-PAGE and silver staining of the gel. The concentration of the complex in a standard
30   solution was determined by measurement of Gla, assuming the presence of nine Gla residues per protein C molecule. Typically, the standard solution was made 0,1% in bovine serum albumin (BSA) and stored in aliquots at -70°C.

Used methods:
35   In the DELPHIA® assay, microtiter plates (Fluor Maxisorp, Nunc) coated with the monoclonal antibody M52 (1 μg/100 μl per well) and incubated overnight at +4°C

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were washed with a wash solution (Wallac) and blocked
with 0,7% BSA in an assay buffer (Wallac) for 2 h. After
washing of the plates, 100 µl of each fraction, with a
dilution of 1:1000 in assay buffer, was added to the
5   wells. The plates were incubated for 1 h on a DELPHIA®
plate shaker (Wallac) and then washed. Eu^{3+} labelled
monoclonal antibodies M11-5 (B and C in figure 1) or
HPC-4 (A in Fig. 1), having a dilution in assay buffer to
a concentration of 20 ng/100 µl, were added, followed by
10   1 h incubation on the shaker. After washing, 200 µl
enhancement solution (Wallac) was added, followed by 5
min incubation on the shaker. Fluorescence was then re-
corded in a DELPHIA® fluorimeter (Wallac).

Surface plasmon resonance (SPR) was measured with
15   the BIAcore® technology (Pharmacia). Streptavidin was
immobilised on the BIAcore® sensorchip CM5 according to
the instructions of the manufacturer. The immobilised
streptavidin gave between 5400 and 6800 response units
(RU). Biotinylated antibody, having a concentration of
20   0,34 mg/ml in 10 mM Tris-HCl/150 mM NaCl (pH 7,75), was
immobilised at a flow rate of 5 µl/min, which resulted in
a response of 2800-3000 RU. Aliquots of protein stock
solution were diluted in flow buffer and 20 µl was in-
jected during the association phase at a constant flow
25   rate of 5 µl/min. The dissociation phase was monitored at
the same flow rate for 10 min. The system was regenerated
by short pulses of 50 mM NaOH at a flow rate of 2 µl/min.
For the fitting of the lines to the experimental data,
see e.g. He, X., Shen, L., Malmborg, A.C., Smith, K.J.,
30   Dahlback, B., Linse, S., Biochemistry, 36:3745-3754
(1997).

Production of monoclonal antibodies:
Balb/c mice were immunised with a mixture of PCI in
complex with PSA (approximately 80%) and PCI cleaved from
35   such a complex (approximately 20%). Each mouse was im-
munised three times with approximately 10 µg of said mix-
ture each time. The two first immunisations were made in-

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tracutaneously with said mixture emulsified in Freund's complete adjuvant, whereas the third immunisation was administered subcutaneously with Freund's incomplete adjuvant. Testing of the mouse plasma, using an ELISA with

5   an appropriate antigen coated in a 96 well microtiter plate, indicated a good antibody response after about two months. Five, four and three days prior to cell fusion, the mice were injected intraperitoneally with approximately 50 µg of the same immunogen (no adjuvant) each

10 time. On cell fusion, the spleen cells were extracted and fused with the myeloma cell line SP2/-Ag14 using 45% polyethylene glycol 1540 and 7% DMSO (dimethyl sulphoxide) under standard conditions (see Borrebäck, C.A.K., Eylar, M.E., *J. Biol. Chem.*, **256**:4723-4725 (1981)). Fused

15 cells in DMEM medium supplemented with HAT (hypoxanthine, aminopterin, thymidine) were seeded into 96 well microtiter plates at a cell density of approximately 10^5 cells per well together with approximately 2×10^4 feeder cells per well. After about ten days, hybridoma supernatants

20 were screened for antibody production.

Clones producing antibodies with interesting properties were identified as described below. The hybridomas were subcloned twice by limiting dilution using 96 well microtiter plates (0,5 to 1 cell per well) using

25 mouse peritoneal macrophages as feeder cells. Stable clones producing monoclonal antibodies of interest were grown to high cell density and injected intraperitoneally into pristane-primed (0,2 ml per mouse) mice for antibody production. Antibodies were also produced on preparative

30 scale by tissue culture (Technomouse®, Integra Biosciences). The antibody was purified from the ascitic fluid/tissue culture medium using chromatography on Protein A Sepharose® according to the instructions of the manufacturer. The cell lines were preserved frozen in 95%

35 calf serum and 5% DMSO in liquid nitrogen.

Procedures for the identification of antibody producing clones that were specific for the complexed/-

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cleaved form of PCI were carefully tailored. Microtiter
plates were coated overnight with rabbit antimouse im-
munoglobulins (DAKO; 1 µg/well in 50 µl) in 0,1 M car-
bonate buffer with pH 9,6. After washing, the wells were

5   "blocked" for 15 min with a solution of bovine serum
albumin (10 mg/ml, 100 µl per well) in 50 mM Tris-HCl,
0,1 M NaCl with pH 7,4. After washing, culture medium was
added to identify antibody producing clones. After wash-
ing again, the wells were incubated with a tracer agent

10 in the above buffer for 1 h. As ^{125}I-labelled tracer
agents, native PCI as well as APC:PCI complexes were
used. The clones were also tested with the same method
and found not to react with APC. After 1 h of incubation
with the tracer agent, the plates were washed with the

15 buffer, after which the radioactivity was measured. By
having APC in the complex labelled with ^{125}I, it was
possible to select antibody producing clones that bound
PCI in complex with APC. Moreover, a comparison with the
binding of labelled, native PCI helped to identify clones

20 that bound PCI in complex with APC with high specific
affinity, but had a low specific affinity for native PCI.
One of the antibodies (M36) with specific affinity
for APC:PCI complexes and cleaved PCI was carefully
characterised. Three other monoclonal antibodies were

25 also used; one against protein C that has been charac-
terised (Ohlin, A.K. and Stenflo, J., *vide supra*), and
two against PCI which do not discriminate between cleaved
and native PCI. One of the latter two antibodies has been
described before (M11-5; Laurell, M., Christensson, A.,

30 Abrahamsson, P-A., Stenflo, J., Lilja, H., J. Clin.
Invest., **89**:1094-1101 (1992)), whereas the other one
(M52) was new (preparation disclosed herein).
Characterisation of monoclonal antibodies:
Several monoclonal antibodies with the appropriate

35 properties were produced. Of these, M36 was carefully
characterised. This monoclonal antibody was immobilised
on an Affi Gel® 10 column (Biorad) and APC-complexed (A),

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cleaved (B) and native (C) PCI were chromatographed on
the column, as is shown in Fig. 1. The antibody bound to
both cleaved and complexed PCI with high affinity, but
was found not to bind or even retard the native PCI.

5   The dissociation constants of the antibody for its
binding to PCI in complex with APC, cleaved PCI and
native PCI, respectively, were measured with standard
plasmon resonance technique. The dissociation constant,
Kd, of M36 was 4×10^{-10} M for PCI in complex with APC and

10   2×10^{-10} M for the cleaved PCI, respectively, as is shown
in Fig. 2. The value of Kd for the native PCI was too
low, i.e. $\geq 10^{-5}$ M, to be determined accurately (not shown
in Fig. 2). In summary, these results established that
the selected monoclonal antibody M36 binds complexed PCI

15   and cleaved PCI with very high affinity, but has very
low, if any, affinity for native PCI.

         As a tracer agent, a monoclonal antibody against
protein C was used. This antibody, which was made by
fusion of spleen cells with the myeloma cell line NS 1,

20   is described by Ohlin, A.K. and Stenflo, J. (vide supra).
Its epitope is in the calcium-binding N-terminal EGF-like
domain of protein C, a domain exposed on both protein C
and APC. Furthermore, this antibody binds to protein C
and APC with high affinity, but the dissociation constant

25   has not been quantitatively measured. The antibody binds
to said epitope in a calcium-dependent manner, and it
should be stated that no special requirements are made on
this antibody, except that it should bind complexed APC
with high affinity and can be labelled without any loss

30   of affinity for the antigen. Among the protein C anti-
bodies tested, this one worked best.
         Collection of blood:
         Blood was collected in 5 ml siliconised glass vacuum
tubes (Becton Dickinson) containing 0,5 ml of a 0,129 M

35   trisodium citrate solution, to which benzamidine had been
added to a concentration of 50 mM. Usually, the samples
were centrifuged within 4 h after the blood was collec-

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ted, and the plasma was either assayed immediately or frozen at $-70°C$. The benzamidine greatly reduces the rate of complex formation between PCI and proteinases, such as kallikreine.

5   Performance of the assay:

The catching antibody (M36) was biotinylated with a N-hydroxysuccinimide derivative of biotin (NHS-LC-Biotin®; Pierce) according to the instructions of the manufacturer. The biotinylated protein was dialysed against a

10   0,1 M sodium fosfate buffer (pH 7,0) containing 0,1% NaN_3, and it was typically stored at a concentration of 0,89 mg/ml at $+4°C$.

To avoid the use of radioactivity and the need for enzyme conjugation of the tracer agent, i.e. HPC-4, a so-

15   called DELPHIA® method was used. In said method, an Eu^{3+} labelled tracer agent is utilised, and in this assay, the DELPHIA® Eu-labelling kit 1244-302 (Wallac) was used. The HPC-4 antibody can be heavily labelled with up to 80 Eu^{3+} ions per antibody molecule without any loss of affinity

20   for protein C. The tracer agent was stored as frozen aliquots at $-20°C$ at a concentration of 0,043 mg/ml. Even if the DELPHIA® procedure was used in the present investigation, any commonly used means of labelling the tracer agent can probably be used.

25   The biotinylated M36 antibody was diluted with assay buffer (Wallac assay buffer: 50 mM Tris-HCl, 0,9% NaCl, 0,05% NaN_3, 0,01% Tween 40, 0,05% bovine immunoglobulin, 0,5 µM DPTA and 20 µg/ml cherry red) to a concentration of 2 µg/ml prior to use. The samples were analysed in

30   duplicate. 50 µl of buffer blank, standard samples, control samples, plasma blanks and plasma (patient) samples, respectively, were added to the wells of a 96 well microtiter plate (Sero-Wel, Bibby Sterlin Ltd.) followed by addition of 50 µl of the biotinylated M36 antibody to the

35   standard, control and plasma samples, whereas 50 µl of assay buffer was added to the other samples. The plates were then put on a LKB, Wallac shaking device for 30 s

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followed by incubation at room temperature for 80 min.
The entire sample was then transferred to a streptavidine
coated plate (Microtitration strips, DELPHIA®, Wallac),
after which the plate was shaken for 60 min on the sha-
5   king device, followed by washing in a plate washer (LKB,
Wallac). 100 µl of the tracer agent solution was then
added (20 ng/100 µl per well, diluted in assay buffer),
followed by incubation on the shaking device for another
40 min at room temperature. After washing (Wallac wash
10   fluid; Tris buffer, salt and Tween 20, pH 7,8), 200 µl of
an enhancer solution (Wallac enhancement solution) was
added to each well, and the plate was shaken on the sha-
king device for 10 min. Fluorescence was determined in an
LKB-Wallac fluorimeter, and the APC:PCI complex formation
15   was calculated by use of the program MultiCalc (Wallac).
Here, it should be emphasized that the method according
to the present invention gives a linear dose-response
curve, as expected for a very high affinity antibody in a
sandwich-type assay (figure 3).
20   In an alternative to the above procedure, the sam-
ples can be assayed directly on the streptavidine coated
plate together with a biotinylated antibody, thereby a-
voiding the first incubation in a 96 well microtiter
plate. In this manner, an even quicker procedure could be
25   achieved without loss of performance. The time for the
incubation with tracer can also be reduced. Such an al-
teration does not lead to any loss in the performance of
the assay.
          It should be noted that the standard curves are i-
30   dentical when made in buffer and in plasma, thus corro-
borating that the uncleaved inhibitor does not influence
the assay. The signal given by APC:PCI complexes prior to
the addition of standard has been subtracted (Fig. 3).

20

CLAIMS

1. A monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, said monoclonal antibody having specific affinity for both

  i) a complex between a serine proteinase and an inhibitor thereof, and

  ii) a cleaved form of said inhibitor,

while having substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or a derivative thereof having the same biological activity.

2. A monoclonal antibody according to claim 1, wherein said monoclonal antibody is obtainable by immunisation of an animal with a mixture of

  i) a complex between a serine proteinase and an inhibitor thereof, and

  ii) a cleaved form of said inhibitor,

followed by screening for and isolation of said monoclonal antibody.

3. A monoclonal antibody according to claim 2, wherein said animal is a mouse, preferably a Balb/c mouse.

4. A monoclonal antibody according to any one of the preceding claims, wherein said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGK1 and prostatic specific antigen (PSA).

5. A monoclonal antibody according to any one of the preceding claims, wherein said inhibitor is protein C inhibitor (PCI) or α_1-antitrypsin.

6. A method for preparation of a monoclonal antibody as defined in any one of claims 1-5, wherein an animal is immunised with a mixture of

  i) a complex between a serine proteinase and

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an inhibitor thereof, and

ii) a cleaved form of said inhibitor,
followed by screening for and isolation of said mono-
clonal antibody.

7. A method for preparation of a monoclonal antibody
according to claim 6, wherein said animal is a mouse,
preferably a Balb/c mouse.

8. A method for monitoring the activity of systems
involving protein C inhibitor, wherein a monoclonal anti-
body as defined in any one of claims 1-5 is used in an
immunoassay.

9. A method according to claim 8, wherein said im-
munoassay comprises a sandwich-type immunoassay.

10. A method according to claim 9, wherein said
sandwich-type immunoassay is a technique comprising a
tracer agent and said monoclonal antibody bound to a sur-
face.

11. A method according to claim 10, wherein said
tracer agent comprises an antibody having specific
affinity for said serine proteinase or an epitope shared
by said serine proteinase and said inhibitor.

12. A method according to claim 11, wherein said
tracer agent is conjugated to a suitable enzyme and/or
labelled with a tracing substance.

13. A method according to claim 12, wherein said
enzyme is an alkaline phosphatase, horse radish per-
oxidase or a β-galactosidase.

14. A method according to claim 13, wherein said
tracing substance is ^{125}I, ^{131}I, Eu^{3+} or Sm^{3+} or a similar
lanthanide.

15. A method for diagnosis of venous thrombosis,
arterial thrombosis, embolism, coronary infarction,
disseminated intravascular coagulation or disorders
involving lupus anticoagulants, wherein a monoclonal
antibody according to any one of claims 1-5 is utilised.

16. A method for diagnosis of venous thrombosis,
arterial thrombosis, embolism, coronary infarction,

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disseminated intravascular coagulation or disorders in-
volving lupus anticoagulants, wherein a method according
to any one of claims 8-14 is utilised.

     17. Use of a monoclonal antibody according to any
one of claims 1-5 for in vitro diagnosis of venous throm-
bosis, arterial thrombosis, embolism, coronary infarc-
tion, disseminated intravascular coagulation or disorders
involving lupus anticoagulants.

     18. Use of a method according to any one of claims
8-14 for in vitro diagnosis of venous thrombosis, ar-
terial thrombosis, embolism, coronary infarction, dis-
seminated intravascular coagulation or disorders in-
volving lupus anticoagulants.

     19. A kit for qualitative or quantitative deter-
mination of the activity of systems involving protein C
inhibitor comprising a monoclonal antibody according to
any one of claims 1-5.

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ABSTRACT

The present invention relates to a monoclonal anti-
body suitable for monitoring the activity of systems in-
5   volving protein C inhibitor, a method for preparation of
said monoclonal antibody, a method for monitoring the
activity of systems involving protein C inhibitor and a
method for diagnosis of *e.g.* venous thrombosis, wherein
said monoclonal antibody is utilised. Said monoclonal
10   antibody is suitable for monitoring the activity of
systems involving protein C inhibitor, and it has speci-
fic affinity for both

         i)   a complex between a serine proteinase and
              an inhibitor thereof, and

15   *ii)* a cleaved form of said inhibitor,
but has substantially no specific affinity for said
inhibitor in its uncleaved and uncomplexed form;
or a derivative thereof having the same biological
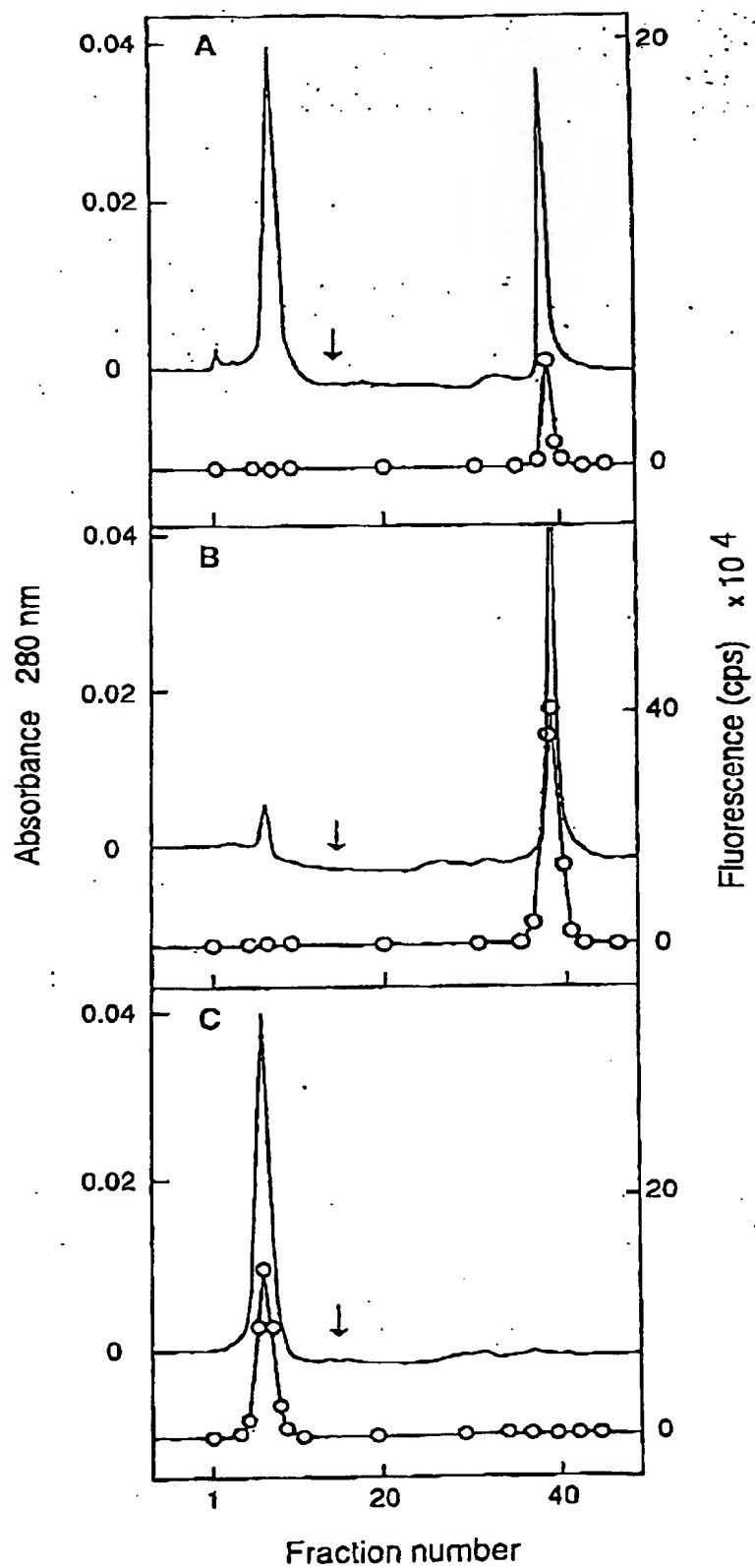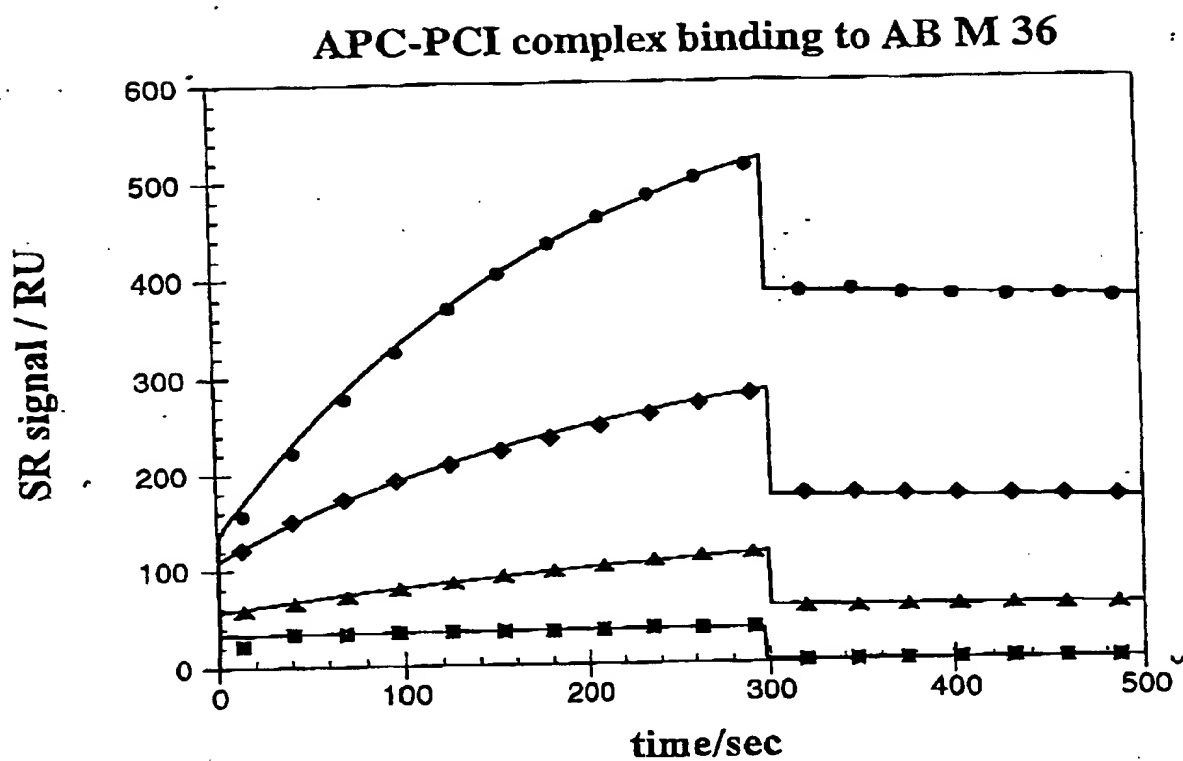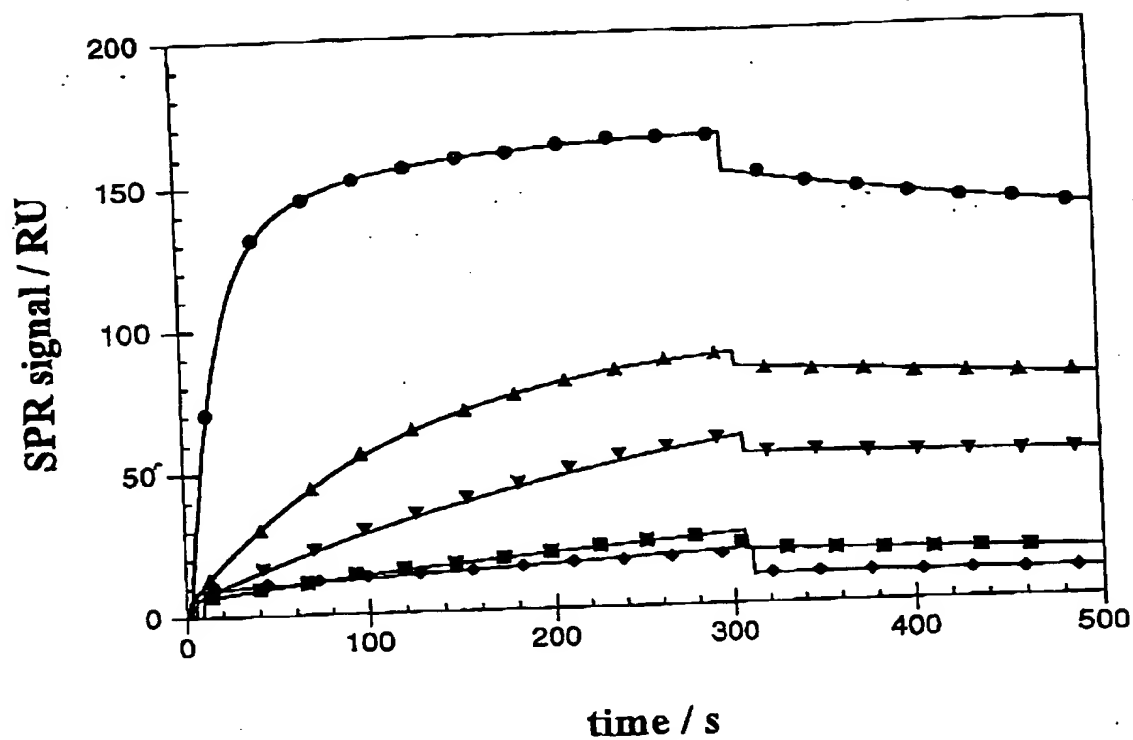activity.

20

Figure 1

APC-PCI complex binding to AB M 36

Figure 2A

den PCI binding to Ab M36

Figure 2B

Figure 3